ONLINE SUPPLEMENT

METHODS

Ethical policies
The investigation for animals handling was performed in agreement with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes (authorization of the laboratory # 00577). Human subjects gave informed consent that was approved by an institutional review committee.

Chemicals
18α-glycyrrhetinic acid, flufenamic acid, carbenoxolone, probenecid, mefloquine (QUO24-1, Bioblocks, CA, USA). Reactive Blue-2 (RB-2) was from Tebu (Le Perray-en-Yvelines, France). Antagonists, NF449, MRS2578, MRS2179, ARL67156 and suramin were from Tocris Bioscience (R&D Systems Europe, Lille, France). Other compounds were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Stock solutions of drugs were prepared according to the manufacturers' protocol, and appropriate vehicle controls were used. The effect of pharmacological blockers was tested after an incubation period of at least 15 minutes vs control vehicle.

Animals
Mice genetically invalidated for $P2Y_6$ (*P2ry6*$^{-/-}$), $P2Y_2$ (*P2ry2*$^{-/-}$), P2X7 (*P2rx7*$^{-/-}$), pannexin1 (*panx1*$^{-/-}$), connexin37 (*Cx37*$^{-/-}$) [1] and connexin43 (*Cx43*$^{+/-}$) were generated as previously described [2-4]. All animals were manipulated in accordance with the European Community Standards on the Care and Use of Laboratory Animals (Ministère de l'Agriculture, France, authorization No. 6422). The protocol was approved by the Committee on the Ethics of Animal Experiments of "Pays de la Loire" (permit # CEEA.2011.14).

Quantitative real time reverse transcription-polymerase chain reaction (RT-PCR) analysis
Mesenteric arterial field were dissected in ice cold PSS, dried and stored at -20°C in RNAlater Stabilization Reagent (Qiagen). RNA extraction was performed using the RNeasy® micro kit (Qiagen). 100 ng of RNA extract were used to synthesize cDNA using the QuantiTect® Reverse Transcription kit (Qiagen). Quantitative real-time PCR was performed with Sybr® Select Master Mix (Applied Biosystems) using a Light cycler 480 Real-Time PCR System (Roche). Primer pairs were designed using primer 3 and those presenting a single peak of dissociation and an efficacy ranging from 1.85 to 2.1 were retained (**Table 1**). Gapdh, Gusb and Hprt were used as housekeeping genes for normalisation. Results were expressed as: $2^{(Ct\ target\ gene - Ct\ mean\ of\ ref\ genes)}$.

Animal model of heart failure
Heart failure (HF) was induced by experimental myocardial infarction. Preoperative analgesic buprenorphine injection (Temgesic® 0.1mg/kg subcutaneous) was performed. Animals were anaesthetized by intraperitoneal injection of a mixture of ketamine (50 mg/kg) and Xylazine (6 mg/kg) and intubated-ventilated at 120-130 breaths per minute (tidal volume 200 µL). Myocardial infarction was performed by permanent ligation of the left anterior descending coronary artery. In sham-operated

controls, the thorax and pericardium were opened, but no ligation was performed. After either procedure, the chest was closed and the mice were extubated and allow recovering on spontaneous respiration. After 6 to 8 weeks, mice developed altered left ventricular dilatation and reduced shortening fraction.

Functional analysis of MRA

Animals were sacrificed by CO_2 inhalation. Mesenteric arteries were dissected in ice-cold physiological salt solution (PSS) of the following composition (mmol/L): 130.0, NaCl; 15.0, $NaHCO_3$; 3.7, KCl; 1.6, $CaCl_2$; 1.2, $MgSO_4$ and 11.0, glucose. Pharmacological study was performed on 2-mm-long arterial segments mounted on a wire-myograph (DMT, Aarhus, DK) [5]. Cumulative concentration-dependent contraction was tested on arteries with a disrupted endothelium (flushed with 1ml Triton X-100 0.04 %). Cumulative concentration-response curve to acetylcholine was performed on arteries contracted with phenylephrine (1 µM). Endothelium-independent relaxation was tested at the end of the protocol in response to the nitric oxide donor, sodium-nitroprusside (SNP).

For pressure myography, third order mesenteric arteries (internal diameter 140-220 µm) were cannulated between two glass pipettes and bathed in PSS (pH 7.4, PO_2 160 mmHg, and PCO_2 37 mmHg). Pressure was controlled by a servo-perfusion system and diameter changes and arterial wall thickness were measured continuously. MT was determined in response to stepwise increases in intra luminal pressure from 10 to 125 mmHg using a video-monitored perfusion system (LSI) [6]. At each pressure, a 5-6 min-equilibration period was allowed to achieve stable vessel diameter. The experiment was repeated with Ca^{2+}-free PSS containing ethylenbis-(oxyethylenenitrolo) tetra-acetic acid EGTA (2 mM), the non specific phosphodiesterases inhibitor papaverin (100 µmol/L) and sodium nitroprusside (10 µM) to ensure complete arterial relaxation, and the passive diameter was recorded for each pressure. MT at a given perfusion pressure was defined as the magnitude of the percent myogenic tone (%MT) at that pressure. The %MT was expressed by the active (AD) and passive vessel diameters (PD) such that $\%MT = [(PD - AD)/PD] \cdot 100\%$.

Calcium mobilization assay

Early passages (P3-4) VSMC cultures prepared from enzymatically digested mesenteric arteries were used for in vitro experiments [7]. Cells were cultured in DMEM supplemented with 10% FBS and antibiotics. Intracellular calcium mobilization was measured on Fura-2-acetoxymethyl ester (Invitrogen) loaded cells. Dual excitation at 340/380 nm with single emission at 510 nm was assessed using a Flexstation-3 Microplate Reader (Molecular Devices). Data represent area under the curve of the ratio 340/380 signal normalized to maximal signal obtained by cells permeabilization with 0.1% saponine.

Freshly dissociated cells were used to avoid any bias linked to cell culturing. Smooth muscle cells were dissociated from arteries using a HEPES-buffered isolation solution containing: (in mM) 140 NaCl, 80 sodium glutamate, 5.6 KCl, 2 MgCl2, 10 HEPES, and 10 glucose (pH 7.35 with NaOH). Briefly, mesenteric arteries were placed into isolation solution containing 1 mg/ml papain, 1 mg/ml dithioerythreitol and 1 mg/ml bovine serum albumin (BSA) for 25 minutes at 37°C. Arteries were then immediately transferred to isolation solution containing 0,7 mg/ml collagenase F and 0,3 mg/ml collagenase H (Sigma), 100 µM CaCl2 and 1 mg/ml BSA for 8 minutes at 37°C. Arteries were subsequently washed in isolation solution and dispersed using a

pipette to yield single smooth muscle cells. Cells were allowed to adhere in 96 well micro plate coated with type I rat-tail collagen (Santa Cruz) in DMEM cultured medium containing 10% FBS and antibiotics for one night. Intracellular calcium mobilization was measured as described above.

Measurement of GTP-bound RhoA

RhoA activation was assessed in agonists-stimulated aorta. Thoracic aortas were dissected from perivascular fat and adventitial tissue in icecold PSS and the endothelium was disrupted by perfusion of PSS containing Triton X-100 (0.04 %, 1mL). Aortic rings (2 mm) were stimulated for 10 min in 37°C heated PSS containing nucleotides or the thromboxane A_2 analogue U46619. Tissues were snap frozen in liquid nitrogen, reduced to powder and resuspended in ice-cold homogenization buffer. GTP-bound RhoA content was determined with using G-LISA™ (Cytoskeleton, Denver, CO) according to the manufacturer instructions.

Western Blot

Smooth muscle cells were obtained from the whole mesentery arterial bed. After removing adventitial peripheral fat with thin forceps MRA were enzymatiquely digested using elastase (0.125 U/ml) and collagenase (2 U/ml) (Worthington, Lakewood NJ) over night at 37°C with agitation in DMEM medium without serum. Cells between P2 and P4 were seeded in 48 well plates and starved once reached 80% of confluence for 12h. Stimulation was performed for 3 min at 37°C stopped with ice cold PBS and directly lysed and homogenized in loadding Buffer: 1% SDS, 10 mmol/L Tris, 1mmol/L Sodium orthovanadate 1 mmol/L, Sodium fluoride 10 mmol/L, β-glycerophosphate 10 mmol/L, complete protease inhibitor cocktail (Roche), 5% β-mercapto ethanol. After boiling 5 min at 90°C, proteins were separated by SDS-PAGE (12% acrylamide) and transferred to nitrocellulose membranes (GE Healthcare). Membranes were analyzed for phosphorylated proteins using the appropriated antibodies (**Table2**).

Statistical analyses

Data are presented as mean ± SEM. Statistical analyses were performed using Graphpad PRISM (La Jolla, CA, USA). Differences between groups were assessed using two-way ANOVA followed by Fisher's LSD Multiple-Comparison Test except for single dose of inhibitors comparison where one-way ANOVA was used. P values <0.05 were considered statistically significant.

Table 1

Gene	Protein	Gene ID	Amplicon size	Primers sequence	
				forward	reverse
Gapdh	**GAPDH**	NM_008084.2	121	ccggggctggcattgctctc	ggggtgggtggtccagggtt
Gusb	**GUSB**	NM_010368.1	72	ctctggtggccttacctgat	cagttgttgtcaccttcacctc
Hprt	**HPRT**	NM_013556.2	125	ggggtgggtggtccagggtt	aagacattctttccagttaaagttgag
Gja4	**Connexin37**	NM_008120	59	tcctgggaaaaagcactgat	ctgtgtctgtccaggtgacg
Gja1	**Connexin43**	NM_010288.3	86	tcctttgacttcagcctcca	ccatgtctgggcacctct
Gja5 ter	**Connexin40**	NM_008121.2	64	acaggagttctggtgaacagg	ctagcaggcgagtcaggaag
Gjc1 (Gja7)	**Connexin45**	NM_001159382.1	70	acaggagttctggtgaacagg	ctagcaggcgagtcaggaag
P2rx1	**P2X1**	NM_008771.3	86	ccgaagccttgctgagaa	ggtttgcagtgccgtacat
P2rx2	**P2X2**	NM_153400.3	77	agtcagcatcatcaccagga	ctgtgaaccctcatgctctct
P2rx3	**P2X3**	NM_145526	77	catcattcccaccatcatca	aggatgatgtcacagagaacagtc
P2rx4	**P2X4**	NM_011026.2	89	ccaacacttctcagcttggat	tggtcatgatgaagagggagt
P2rx5	**P2X5**	NM_033321.3	77	cacagtcatcaacattggttcc	aggtagataagtaccaggtcacagaag
P2rx6	**P2X6**	NM_011028.2	64	tgtccccagtactccttcca	caccagtgattggctgtcc
P2rx7	**P2X7**	NM_011027.2	76	ctggttttcggcactgga	ccaaagtaggacagggtgga
P2ry1	**P2Y1**	NM_008772.4	60	ctgtgtggaccccattcttt	tcgggacagtctccttctga
P2ry2	**P2Y2**	NM_008773.3	127	tgcgctgatgaactttgttt	ggcaggaaacaggaagaaca
P2ry4	**P2Y4**	NM_020621.3	114	gaagaagcagcagaacacca	caaggagtctgcactggtca
P2ry6	**P2Y6**	NM_183168.1	80	tcttccatcttgcatgagaca	ggatggtgccattgtcct
P2ry12	**P2Y12**	NM_027571.3	94	gagacactcatatccttcagattcag	tcgggacagtctccttctga
P2ry13	**P2Y13**	NM_028808.3	60	atgtgtgagatggggaaagg	gtcccaggggagaaggtg
P2ry14	**P2Y14**	NM_001008497.2	59	ctttgcctccagaggtgaga	ggctggtttggagggtct
Panx1	**pannexin-1**	NM_019482.2	61	agaccaagggagaggacca	gctgctcaggtccaaatctt
Panx2	**pannexin-2**	NM_001002005.2	122	gtacccccctgccctacca	gatttccttctcggggagtg
Panx3	**pannexin-3**	NM_172454.2	63	gaaatctctctggcctcacaa	atacatggccacagccaga

Table 2

Target protein	Reference	Provider
Phospho-p38 (Thr180/Tyr182)	4511	Cell Signaling Technology
p38 MAPK	9212	Cell Signaling Technology
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	9101	Cell Signaling Technology
ERK1	610031	BD biosciences
Phospho-Akt (Ser473)	4060	Cell Signaling Technology
Akt (pan) (C67E7)	4691	Cell Signaling Technology
Phospho-MYPT-1 (Thr850)	04-773	Merck Millipore
Phospho MYPT-1 (Thr 696)	sc-17556	Santa Cruz Biotechnology
MYPT-1	612164	BD biosciences
Phospho-SAPK/JNK (Thr183/Tyr185)	9251	Cell Signaling Technology
JNK1	551197	BD biosciences
Phospho-Myosin Light Chain 2 (Thr18/Ser19)	3674	Cell Signaling Technology
Myosin Light Chain 2	3672	Cell Signaling Technology
Beta-Actin	A5316	SIGMA-ALDRICH

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